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Review

High resolution two-dimensional electrophoresis of human urinary proteins

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Abstract

The use of high resolution two-dimensional electrophoresis (2-DE) for analysis of human urine is reviewed with particular reference to methodological developments, urinary protein mapping and clinical applications. The methodological aspects relate to sample collection and storage, desalting and concentration of the urinary proteins and the choice of 2-DE and detection method. Urinary protein mapping includes the source of the proteins and the identification of their 2-DE positional co-ordinates. The clinical applications include 2-DE of urine from patients with renal disease, Bence Jones proteinuria, cancers of the urogenital tract, nephrolithiasis, cadmium exposure, rheumatoid arthritis, Duchenne muscular dystrophy and pregnancy-induced hypertension. Recent developments, including proteome analysis, are briefly discussed.

Keywords: Two-dimensional electrophoresis; Urine; Urinary proteins; Renal disease; Bence Jones proteinuria; Nephrolithiasis

1. Introduction

Urine contains trace amounts of protein originating from blood plasma, the kidneys and the urogenital tract [1]. Excessive protein in urine ("proteinuria") is usually indicative of disease and can be classified in various ways [2]. In pre-renal proteinuria excessive protein in the blood (from induced synthesis or tissue damage) "overflows" into the urine as a result of overloading the reabsorption capacity of the renal tubules, e.g. Bence Jones proteinuria (BJP) in plasma cell dyscrasias. Renal proteinuria implies kidney damage and can be of a glomerular, tubular or glomerular/tubular-mixed type depending upon its association with glomerular or tubular dysfunction, or both [2]. Whilst glomerular proteinuria is of high protein concentration and shows a predominance of albumin and high molecular weight plasma proteins, tubular proteinuria is of relatively low protein concentration and is characterised by the presence of specific low molecular weight proteins (α_1-microglobulin, retinol binding protein and β_2-microglobulin). In practice, most renal proteinurias are of the mixed type and display a wide distribution of protein content. Post-renal proteinuria is associated with disorders of the urogenital tract (e.g. inflammation/infection and bleeding) resulting in the addition of protein to urine after its formation in the kidneys [2]. The correlation between proteinuria and disease is complex as biological variables (e.g. posture, exercise and pregnancy) induce a glomerular-type physiological proteinuria in healthy individuals. Furthermore, pre-renal proteinuria commonly results in kidney disease leading to renal proteinuria. Nevertheless, urine has enormous

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potential for non-invasive detection of protein biomarkers for the diagnosis and clinical monitoring of human disease.

Electrophoresis is routinely used for protein analysis of human urine in health and disease [2,3]. High resolution two-dimensional electrophoresis (2-DE) [4] is the most powerful protein separation technique currently available and combines two independent electrophoretic parameters (isoelectric point, pI and relative molecular mass, M_r) to "fingerprint" the polypeptide constituents of a complex protein mixture. The present report reviews the application of 2-DE to human urine in health and disease.

2. Methodology

2.1. Sample collection and processing

Urine is a complex biological mixture which shows a circadian variation in composition. Samples often contain particulate matter consisting of salts, casts and micro-organisms [2]. Thus, sample collection and processing need to be standardised. Early morning "mid stream" samples are probably optimal for protein analysis and, ideally, should be freshly collected into sterile tubes containing protease inhibitors followed by centrifugation and denaturation of the native proteins into their constituent polypeptides. In practice clinical samples are usually collected over 24 h in vessels containing a standard amount of preservative [2]. Thus, sample volume dictates the final preservative concentration and individual samples may be exposed to different temperatures resulting, potentially, in varying degrees of proteolysis. The variation in the protein content of the samples may necessitate different degrees of processing (e.g. extent of concentration) resulting in differential loss of protein. Thus, standardisation of collection and processing is a major problem and inter-laboratory comparison of published data is difficult. Within an individual study it is essential that all urines are handled in an identical manner. To facilitate batch comparison, we recommend that individual urines be freshly collected and centrifuged prior to storage at −70°C. Upon thawing, the samples should be thoroughly mixed but not recentrifuged as this results in protein loss [5]. Subsequent processing designed to desalt and concentrate the urinary proteins is a matter of choice – the recommended methods are summarised in Table 1. Anderson et al. [1] dialysed and lyophilised the urine prior to gel filtration (P-4 Bio-Gel) and lyophilisation of the excluded peak which was resolubilised in a sample denaturing solution. The potential loss of Tamm–Horsfall mucoprotein and some of the globulins was acknowledged [1]. Clark et al. [6] concentrated dialysed urine by dialysis against 40% (w/v) polyethylene glycol (PEG) 6000. Frearson et al. [7]

Table 1
Chronological development of methodology for 2-DE of human urinary proteins

Authors (Reference)	Desalting/concentration	2-DE	Protein load	Detection
Anderson et al. [1]	Dialysis, gel filtration, lyophilisation	ISO-DALT	1–2 mg	CBB[a]
Clark et al. [6]	Dialysis, PEG[b]	ISO-DALT	NS[c]	CBB
Frearson et al. [7]	Dialysis and lyophilisation (X 2)	ISO-DALT	NS	CBB
Marshall et al. [8]	Untreated	Simplified	2.5 μg	Silver
Guevara et al. [14]	Acidified acetone extraction, gel filtration, lyophilisation	ISO-DALT	75 μg	Silver
Gianazza et al. [15]	Dialysis and lyophilisation	IPG-DALT	0.2–1.2 mg	Silver
Gomo et al. [16]	UF[d] (combined systems)	ISO-DALT	10–40 μg	Silver
Tracy et al. [17]	HPLC[e], lyophilisation	ISO-DALT	20–30 μg	Silver
Myrick et al. [18]	Centricon UF	ISO-DALT	40 μg	Silver
Marshall and Williams [13]	Dye precipitation	Simplified	125 μg	CBB

[a]Coomassie Brilliant Blue.
[b]Polyethylene glycol.
[c]Not specified.
[d]Ultrafiltration.
[e]High performance liquid chromatography.

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staining [9,10] to avoi
of urine prior to 2-DE b
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evidence to suggest t
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In our experience, it i
proteinuric urines con
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[8,13,15].

2.2. Two-dimensional

2-DE involves is
dimension) followed
polyacrylamide gel el
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electrophoresis (NEP
by SDS–PAGE for a
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ISO-DALT system d

recommended two consecutive cycles of dialysis and freeze-drying but acknowledged that the use of thymol as a preservative may cause protein loss by precipitation. Marshall et al. [8] used ultrasensitive silver staining [9,10] to avoid the need for the processing of urine prior to 2-DE but subsequently recommended dye precipitation as a simple and novel method for desalting/concentrating urinary proteins prior to 2-DE/CBB staining [11–13]. Guevara et al. [14] used acidified acetone to extract the protein from lyophilised urine prior to chromatography on Bio-Gel P-6. The authors acknowledged the possibility of protein degradation through cleavage of acid-labile linkages [14]. Gianazza et al. [15] recommended only a single cycle of dialysis and lyophilisation prior to 2-DE using immobilised pH gradients to eliminate cathodic drift. Gomo et al. [16] used a combination of commercial ultrafiltration systems to desalt and concentrate urine 1000-fold. Tracy et al. [17] resorted to high pressure liquid chromatography (on Fracto-Gel HW-40-F) followed by lyophilisation whilst Myrick et al. [18] recommended Centricon-10 ultrafiltration units (Amicon) to simultaneously desalt and concentrate urinary proteins.

There has been no direct comparison of the effects of the above methods upon the recovery and 2-DE patterns of human urinary proteins but there is much evidence to suggest that dialysis, ultrafiltration and freeze-drying (particularly as an intermediate step) leads to protein loss [1,8,19–21]. The desalting and concentration of urine is tedious, time-consuming, expensive and unsuitable for high sample throughput. In our experience, it is unnecessary prior to 2-DE as proteinuric urines containing >0.5 g/l protein can be directly analysed by 2-DE/CBB staining and urines of lower protein content by 2-DE/silver staining [8,13,15].

2.2. Two-dimensional electrophoresis (2-DE)

2-DE involves isoelectric focusing (IEF, first dimension) followed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE, second dimension) [4] or non-equilibrium pH gradient electrophoresis (NEPHGE, first dimension) followed by SDS–PAGE for analysis of the more basic polypeptides [22]. Anderson et al. [23,24] introduced the ISO-DALT system designed to simultaneously cast, load and run upto 20 IEF gels followed by SDS–PAGE in DALT tanks containing 10 slab gels (80 of which could be simultaneously cast). The system was designed to optimise reproducibility and enhance sample throughput. Many authors claim to have used the ISO-DALT system for urinary protein analysis (Table 1) but it is sometimes unclear whether ISO-DALT refers to the Anderson equipment or is simply used as a pseudonym for 2-DE [1,6,7,14,16–18,25–37]. We have adopted a simplified method of 2-DE which allows high protein loads, avoids equilibration (and associated protein loss) and enhances reproducibility by simultaneous processing of upto 16 small and robust (75×75×3 mm) 6–20% (w/v) polyacrylamide gradient gels [8,38]. A major drawback of this system is the use of conventional carrier ampholytes which are prone to cathodic drift resulting in loss of resolution of the basic polypeptides. Contemporary 2-DE methods overcome this problem by using immobilised pH gradients [15,39,40]. Surprisingly, automated systems such as the Phast System have had little impact upon urinary protein analysis by 2-DE except for the work of Lapin et al. [41–44] who used a modified 2-DE approach combining cellulose acetate electrophoresis and SDS–PAGE under non-reducing conditions.

2.3. Detection and identification

The use of Coomassie Brilliant Blue (CBB) for detection of urinary proteins following 2-DE [1,6,7,25–29] has been superseded by silver staining [8,14–18,45–47] (Table 1) although Grover and Resnick [33,37] combine the two. Silver staining is undoubtedly more sensitive than CBB but it poses a number of problems. Silver is less specific than CBB for the detection of protein and the interpretation of silver stained urinary protein 2-DE patterns is often hampered by diffusely staining areas probably due to mucopolysaccharides [13]. In addition, silver staining often reveals extensive horizontal and vertical streaks which interfere with densitometric analysis. Different silver stains have been exploited for 2-DE urinary protein analysis but there has been no direct comparison of the relative merits of these methods for this purpose. There are clear anomalies relating to detection sensitivity – we recommend 2.5 μg of urinary protein [13], whilst Giannazza et al. [15] used upto

1200 μg (most authors recommend 10–75 μg [14,16–18]). This may genuinely reflect variations in silver stain sensitivity but may be partly due to differences in sample processing and the choice of protein assay or 2-DE method. Protein loads based upon freeze dried weight may include insoluble protein, non-proteinaceous material (e.g. glycosoaminoglycans) and residual chromatographic column finings or buffer salts.

Immunoblotting [48] has been widely adopted for detection and identification of urinary proteins following 2-DE [16,27,44,45,47,49–53] and complements the more traditional approach of immunodeletion and co-electrophoresis of purified proteins [1,16,27].

3. Applications

3.1. Urinary protein mapping

2-DE has been widely used to investigate the protein composition of normal urine in order to:

(i) identify the nature and source of the proteins present,
(ii) establish the positional co-ordinates of the polypeptide spots in the form of pattern composites, and
(iii) detect sex-related differences.

Anderson et al. [1] distinguished eight classes of proteins present in urine (plasma, kidney, urogenital, tissue, hormonal, pregnancy-related, tumour-associated and microbial) and demonstrated the application of the ISO-DALT system to healthy male controls and pre- versus post-menopausal women. The reproducibility and compositional variation of the patterns of different individuals was investigated and a reference pattern was compiled defining five landmark areas [1]. Edwards et al. [27] subsequently identified the 2-DE spot positions of transferrin, albumin, haemopexin, α_2-HSglycoprotein, α_1-antitrypsin, GC-globulin, α_1-acidglycoprotein, Znα_2-glycoprotein, retinol binding protein, β_2-microglobulin, the immunoglobulin light chains and the most acid urinary protein (MAUP). Carbamylated creatine kinase and homogenised rabbit psoas muscle were recommended as internal standards for calibration of the positional co-ordinates of the polypeptide spots [27]. In a complementary study Gomo et al. [16] used electroblotting to identify the 2-DE positions of urinary high-density-lipoprotein apolipoproteins (HDL-Apos; A-I, A-II, and C) and their isoforms. Whilst Lapin et al. [41] identified the 2-DE positions of 20 urinary proteins, it is important to acknowledge that the method combined cellulose acetate electrophoresis (first dimension) with SDS–PAGE under non-reducing conditions (second dimension) and consequently the resulting patterns cannot be directly compared with those obtained using conventional high resolution 2-DE [4]. More recently, Wiederkehr et al. [54] used PEG precipitation and Protein-A Sepharose/Protein-G Sepharose affinity chromatography to isolate and map the 2-DE positions of urinary immune complexes and Tracy et al. [17] adopted the ISO-DALT system and landmark strategy of Anderson et al. [1] to detect and differentiate urinary proteins of kidney and plasma origin. In a more comprehensive follow-up study Wiederkehr's group [45] combined co-electrophoresis, immunoblotting and affinity chromatography to identify Tamm–Horsfall mucoprotein, the isoforms of human chorionic gonadotrophin (β chain), subunits of prostatic acid phosphatase, the secretory component of IgA and constant breakdown products of α_1-antitrypsin and retinol binding protein. In addition, they reported three sex-related proteins markedly enhanced in female urine, particularly during pregnancy. Recently, we have recommended dye precipitation of urine for protein concentration prior to 2-DE [13]. Silver staining confirms excellent protein recovery and indicates that the 2-DE positional co-ordinates of most of the polypeptide spots are unaffected (Fig. 1). Urine of normal protein content and distribution can thereby be detected by CBB staining (Fig. 2), which avoids the problems of silver staining (Fig. 1). The patterns reveal over 300 polypeptide spots and, with the exception of albumin, show surprisingly little plasma protein (Fig. 2). There is a disproportionate excess of immunoglobulin light chain (relative to heavy chain) and a characteristic prominence of Tamm–Horsfall mucoprotein, α_1-microglobulin and other urine-associated proteins (Fig. 2(B)).

3.2. Clinical applications

2-DE has been widely applied to human urine to detect protein biomarkers for the diagnosis and clinical monitoring of disease (Table 2). Consequently,

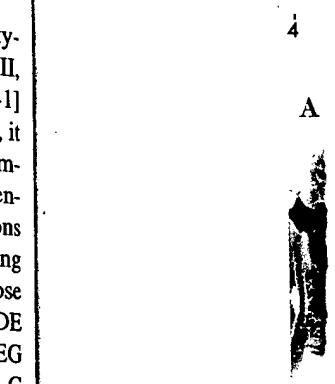

Fig. 1. 2-DE of human urine (2.
of pooled urine (0.07 g/l protein
(v/v) phosphoric acid) and the r
1 ml of acetone (to remove exce
1% (w/v) SDS, 1% (v/v) 2-merc
precipitation is a simple metho
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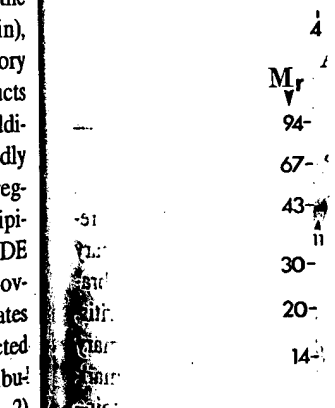

Fig. 2. 2-DE of human plasma
determined by co-electrophores
case; the patterns correspond t
albumin; (2) transferrin; (3) he
α_1-antitrypsin; (9) GC-globulin
(14) Ig λ, κ light chains; (15)
apolipoprotein A-II; (20) Tamn
gonadotropin (hCG) β chain;
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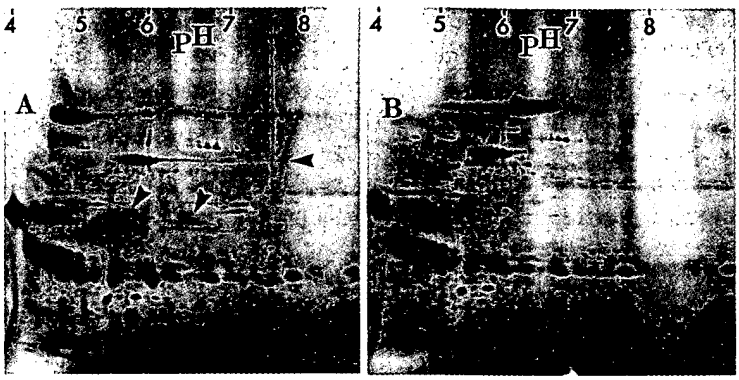

Fig. 1. 2-DE of human urine (2.5 μg protein) before and after dye precipitation (A, B, respectively) as detected by silver staining. Briefly, 1 ml of pooled urine (0.07 g/l protein) was mixed with 0.25 ml of a CBB dye reagent concentrate (0.05% (w/v) CBB in 25% (v/v) ethanol and 25% (v/v) phosphoric acid) and the mixture centrifuged (13 400×*g*, 5 min) to recover the insoluble protein–dye complex which was washed with 1 ml of acetone (to remove excess dye) prior to solubilisation in 20 μl of sample denaturing solution (0.0625 M Tris–HCl pH 6.8 containing 1% (w/v) SDS, 1% (v/v) 2-mercaptoethanol and 20% (w/v) glycerol). Note: (i) silver staining allows 2-DE of non-concentrated urine, (ii) dye precipitation is a simple method for recovery and concentration of urinary protein and eliminates diffusely staining areas (arrowheads, A) probably due to mucopolysaccharides, and (iii) the 2-DE positional co-ordinates of most polypeptides are unaffected by dye precipitation as the protein–dye complex dissociates during IEF.

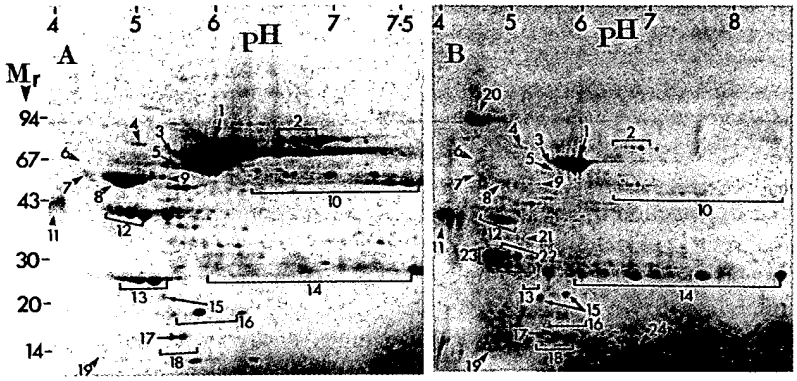

Fig. 2. 2-DE of human plasma (A, 200 μg) and human urine (B, 125 μg) indicating the positions of the major plasma and urinary proteins as determined by co-electrophoresis, immunodeletion, immunoblotting and comparison with published patterns [1,27,30,31,33,45,52,54]. In each case, the patterns correspond to pooled samples analysed under reducing conditions and detected with CBB. The identified proteins are: (1) albumin; (2) transferrin; (3) hemopexin; (4) α_1-β-glycoprotein; (5) Ig α chains; (6) α_1-antichymotrypsin; (7) α_2-HS-glycoprotein (fetuin); (8) α_1-antitrypsin; (9) GC-globulin; (10) Ig γ chains; (11) α_1-acid glycoprotein (orosomucoid); (12) haptoglobin β chain; (13) apolipoprotein A-I; (14) Ig λ, κ light chains; (15) retinol binding protein; (16) haptoglobin α_2 chain; (17) prealbumin; (18) haptoglobin α^{1F} and α^{1S} chains; (19) apolipoprotein A-II; (20) Tamm–Horsfall mucoprotein (uromodulin); (21) Zn–α_2-glycoprotein; (22) α_1-microglobulin; (23) human chorionic gonadotropin (hCG) β chain; and (24) β_2-microglobulin. In this and the subsequent figures, the anode of the IEF gel is to the left and electrophoresis performed from top to bottom. M_r indicates relative molecular mass×10^{-3}.

most publications include at least "pilot studies" demonstrating application of 2-DE to individual samples representing various disease states. However, in the absence of a sufficient number of specimens and/or appropriate controls it is difficult to evaluate the significance of such reports. Studies which have been specifically directed towards particular clinical investigations are summarised in Table 2.

3.2.1. Renal disease

Glomerular dysfunction is characterised by the presence of albumin in urine and high molecular

Table 2
Clinical application of 2-DE to human urine

Disease	Clinical application	Authors (Reference)
Renal disease	Renal failure, pyelonephritis	Clark et al. [28]
	Glomerular disease	Gomo et al. [16], Tracy et al. [17]
	Haemodialysis	Miyata et al. [49]
	Amyloidosis	Miyata et al. [49], Argiles et al. [50]
	Transplantation	Clark et al. [28], Bauer et al. [55]
Myelomatosis	Bence Jones proteinuria	Harrison [30], Harrison [32], Harrison et al. Tichy et al. [46], Stulik et al. [47]
Urogenital cancer	Prostatic carcinoma	Edwards et al. [26], Grover and Resnick [36]
	Bladder squamous cell carcinoma	Celis et al. [51], Rasmussen et al. [52]
Nephrolithiasis	Protein–calcium oxalate binding	Morse and Resnick [68]
	Calcium oxalate stone formers	Grover and Resnick [37]
Cadmium toxicity	Occupational exposure	Marshall et al. [8], Myrick et al. [18,70]
Rheumatoid arthritis	Associated renal function	Clark et al. [6]
Neuromuscular disease	Duchenne muscular dystrophy	Frearson et al. [7]
Pre-eclampsia	Associated renal function	Clark et al. [29]

weight proteins; tubular dysfunction by the detection of low molecular weight proteins including α_1-microglobulin, retinol binding protein and β_2-microglobulin [2]. However, these conditions are not mutually exclusive and consequently, renal proteinuria is usually of a mixed glomerular/tubular-type. In an early study Clark et al. [28] compared the urinary protein 2-DE patterns of patients with hypertensive renal failure, acute renal failure, pyelonephritis and renal transplantation and reported (i) much variation even within groups of similar renal pathology, (ii) a reduction in low molecular weight proteins on recovery following transplantation, and (iii) a predominance of high molecular weight proteins in the proteinuria associated with graft rejection [28]. In a more comprehensive study, Bauer et al. [55] demonstrated complex protein changes between different functional states following renal transplantation. Gomo et al. [16] investigated urinary lipoproteins in glomerular disease using preparative ultracentrifugation and 2-DE/immunoblotting to detect the elevation of HDL-Apos, including unusual isoforms of apo A-I. In a comp hensive study Tracy et al. [17] compared the urin protein patterns of patients with idiopathic memb nous or membranoproliferative glomerulonephri lupus nephritis, diabetic nephropathy and prim systemic amyloidosis using six defined landm areas for reference purposes and identified 34 prote showing a quantitative change in renal disease –, were of plasma origin and seven present in norn urine but not blood plasma or proteinuric uri Miyata et al. [49] used 2-DE/immunoblotting to det abnormal acidic isoforms of β_2-microglobulin in t urine of haemodialysis patients (and the amylo fibrils of haemodialysis-associated amyloidosis) a attributed their formation to advanced glycation e products of the Maillard reaction. In a similar stu Argiles et al. [50] further investigated the amyloid genicity of β_2-microglobulin detecting four or mo isoforms (M_r 12 000; pI 4–6) in amyloid deposits a the urine of kidney donors. The study, which includ amino acid sequencing, indicated that the acid

β_2-microglobulin isoforms were not specific for amyloidosis and the modifications of the β_2-microglobulin did not arise by deamidation (at Asn 17) or limited proteolysis (at the N-terminus) as previously believed [50].

Modified 2-DE methods have also been used for urinary protein analysis in renal disease. Lapin et al. [41] combined cellulose acetate electrophoresis and SDS-PAGE to analyse urine from over 300 patients with renal disease [41–44]. Miyata et al. [57] used non-denaturing native 2-DE to detect a unique spot (pI 7.1–7.2; M_r 23 000) with an amino acid sequence identical to complement factor D in the urine of patients with chronic renal failure. In a recent study Oda et al. [56] used SDS–2-DE to identify a urinary protein (M_r 25 000) from haemodialysis patients as a fragment of perlecan (the protein core of a proteoglycan in the systemic capillary basement membrane).

We have used 2-DE to compare the urinary protein patterns of patients in different stages of renal failure (Fig. 3). Chronic and end stage renal failure are characterised by a progressive increase in the proportion of α_1-microglobulin, retinol binding protein, β_2-microglobulin, apolipoprotein (apo) A-I and an unidentified abnormal cluster of spots (pI 5.5–6.0; M_r 35 000) (Fig. 3). The apo A-I associated with end stage renal failure includes a lower tier of spots as previously reported by Gomo et al. [16]. We have also used a variant of 2-DE involving analysis under non-reducing conditions to compare the urinary protein patterns of hypertension and renal failure (Fig. 4). The method avoids dissociation of high molecular weight (disulphide-linked) oligomeric proteins to yield less

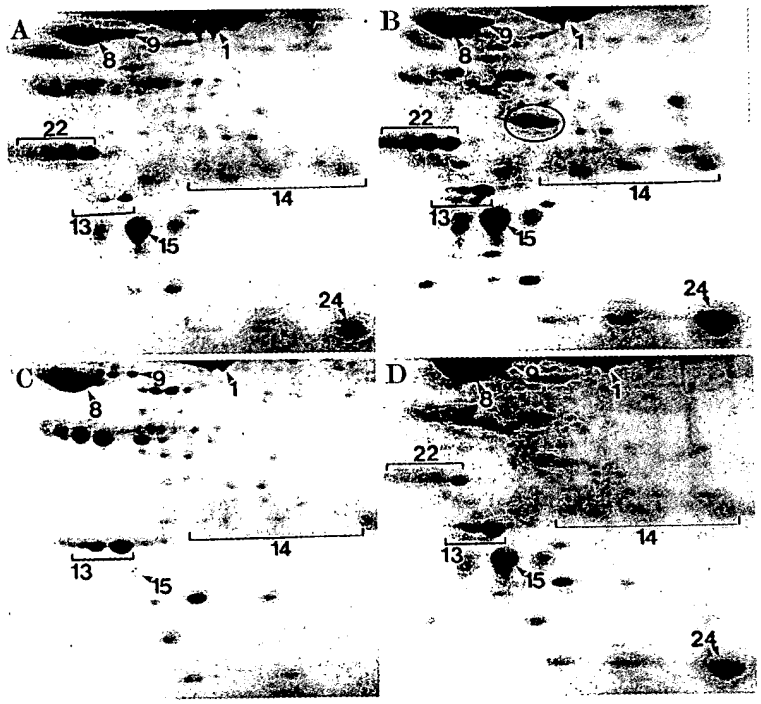

Fig. 3. 2-DE of urine (160 μg protein) from patients with chronic renal failure (A) and end stage renal failure (B) as compared to human plasma ((C), 160 μg protein) and a mix of urine plus plasma (D=B+C). In each case, the patterns correspond to pooled samples (n=9) electrophoresed under reducing conditions and detected with CBB. Only the central gel area of interest is shown. The identified proteins include: (1) albumin; (8) α_1-antitrypsin; (9) GC-globulin; (13) apolipoprotein A-I; (14) Ig λ, κ light chains; (15) retinol binding protein; (22) α_1-microglobulin; and (24) β_2-microglobulin. Note: (i) renal failure is associated with a progressive increase in the proportion of apolipoprotein A-I (including abnormal isoforms), immunoglobulin light chain, retinol binding protein, α_1-microglobulin, β_2-microglobulin and an unidentified cluster of spots (circled, (B)), and (ii) comparison of plasma (C) with plasma plus urine (D) confirms the positional identity of retinol binding protein and apolipoprotein A-I.

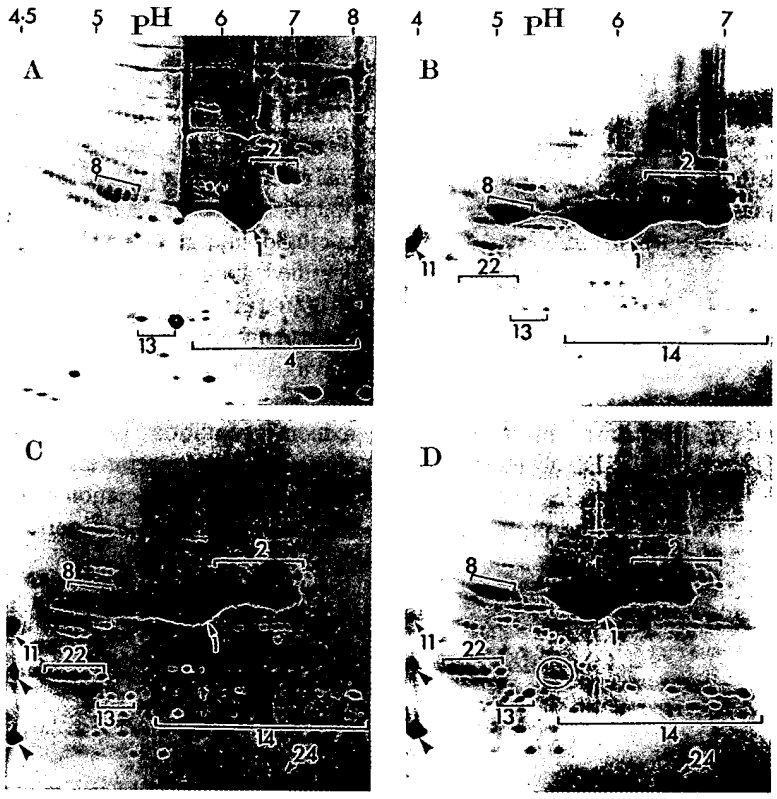

Fig. 4. 2-DE of human plasma ((A), 240 μg protein) and urine (150 μg protein) from patients with hypertension (B), chronic renal failure (C) and end stage renal failure (D). In each case, the patterns correspond to pooled samples (n=6–9) electrophoresed under non-reducing conditions and detected with CBB. The identified proteins include: (1) albumin; (2) transferrin; (8) α_1-antitrypsin; (11), α_1-acid glycoprotein (orosomucoid); (13) apolipoprotein A-I; (14), Ig λ, κ light chains; (22) α_1-microglobulin; and (24) β_2-microglobulin. Note: the protein changes associated with end stage renal failure are consistent with those observed under reducing conditions (c.f. Fig. 3) and include elevation of apolipoprotein A-I (and abnormal isoforms), immunoglobulin free light chain, α_1-microglobulin, β_2-microglobulin and unidentified spots (circled, D; arrowheads, C, D).

complex 2-DE patterns which reflect more closely the selectivity of protein filtration by the kidneys. The patterns confirm the results obtained with conventional 2-DE (Fig. 3) highlighting the progressive increase in the proportion of low molecular weight proteins associated with renal failure but demonstrate more clearly the appearance of abnormal isoforms of apo A-I, and reveal prominent free light chain spots associated with end stage renal failure (Fig. 4).

3.2.2. *Bence Jones proteinuria*

Bence Jones proteinuria (BJP) is an "overload proteinuria" characterised by free immunoglobulin light chain (LC) in the urine. The detection of BJP by immunofixation electrophoresis (IFE) is important in the management of plasma cell dyscrasias [58] as free LC often leads to renal insufficiency ("myeloma kidney") [59] and amyloidosis upon polymerisation/deposition in tissues [60]. LC nephrotoxicity is of particular interest and appears to be related to the physico-chemical characteristics of the LC [58–61]. The advantage of 2-DE over IFE is that it combines two independent electrophoretic parameters thereby clearly identifying the LC constituents as individual spots. Harrison [30] used 2-DE to investigate the LC heterogeneity associated with the "pseudo-oligoclonal" (or "tiger stripe") patterns observed upon IFE with BJP and demonstrated, by immunoblotting, patient-specific heterogeneity [32,34]. This microheterogeneity was similar to that previously observed

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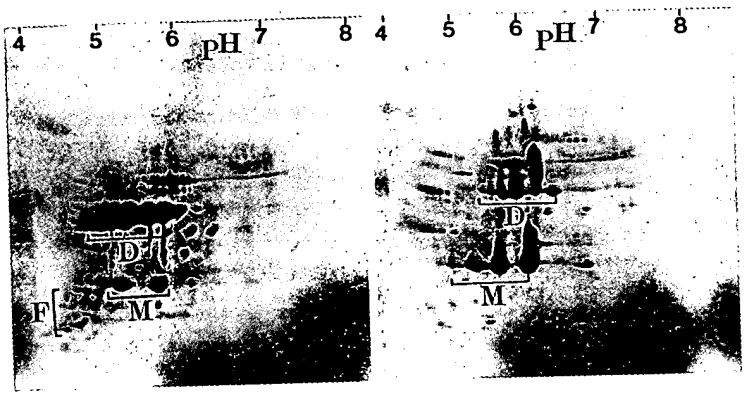

Fig. 5. 2-DE of Bence Jones proteinuria. The patterns correspond to urine (100 μg protein) from two myeloma patients analysed under non-reducing conditions and detected with CBB. Note: (i) the variable proportion of light chain dimer (D), monomer (M) and fragment (F); and (ii) the charge and size heterogeneity of the light chain species.

upon 2-DE of paraproteins [62,63]. Carbamylation failed to fully interconvert the isoforms [32,34] suggesting that a variable content of lysine amino acids does not necessarily explain the charge differences. More recently, Tichy et al. [46,47] studied the LC heterogeneity by immunoblotting following 2-DE in immobilised pH gradients. The analysis of sequential samples from the same patient suggested that the appearance of additional anodal LC isoforms may indicate a poor prognosis. We have used 2-DE under non-reducing conditions to investigate the relative proportion and heterogeneity of LC dimer and monomer in BJP (Fig. 5). The patterns reveal a multiple array of spots arranged in horizontal tiers (indicative of charge heterogeneity) which are sometimes packed close one upon another (indicative of a superimposed size heterogeneity) (Fig. 5). In this case, conventional 2-DE under reducing conditions gives simpler patterns as the dimeric LC is converted into its monomeric form (Fig. 6). However, the patterns still indicate a diverse range of LC heterogeneity characterised by differences in the charge and size of the isoforms and variable amounts of the LC fragments (Fig. 6).

3.2.3. Cancers of the urogenital tract

2-DE of urine has been used to detect marker proteins for the diagnosis and clinical management of urogenital cancers. Edwards et al. [26] detected prostatic cancer antigen 1 (PCA-1; M_r 40 000) in the urine of 16 of 17 patients with prostatic cancer but not in the urine of age-matched controls or patients with testicular cancer, bladder cancer, benign prostatic hyperplasia or non-urogenital cancers. PCA-1 was present in biopsies from malignant and benign hyperplastic prostatic tissue suggesting it is a non-secreted protein of prostatic origin which is released into urine on transition to malignancy [26]. More recently, Grover and Resnick [36] used 2-DE to detect a protein D (pI ~4.0; M_r 22 000 and specifically associated with the prostatic fluid of patients with prostatic carcinoma) as a diffuse string of spots in the urine of patients with prostatic cancer. Celis et al. [51] used 2-DE and immunoblotting in combination with mass spectrometry and microsequencing to analyse the urine of patients with bladder squamous cell carcinomas (SCCs) and detected a calcium-binding protein, psoriasin (pI 6.2; M_r 110 000) which, unfortunately, was also present in 16% of urines from healthy controls and non-SCC bladder tumour patients. In an elegant follow up study they initiated a computerised urinary protein database listing 339 proteins (37% of which have been identified) which can be accessed on the Internet (URL http://biobase.dk/cgi-bin/celis) as a reference for bladder tumour markers [52].

3.2.4. Nephrolithiasis

Kidney stones have an organic matrix (0.5–9% (w/w) of the stone mass) which consists predominantly of proteins from the kidney (Tamm–Horsfall mucoprotein) and blood plasma (albumin and α/β-globulins) [64]. The role of urinary proteins in nephrolithiasis is unclear but it is believed that they exert a protective effect and defective proteins (e.g.

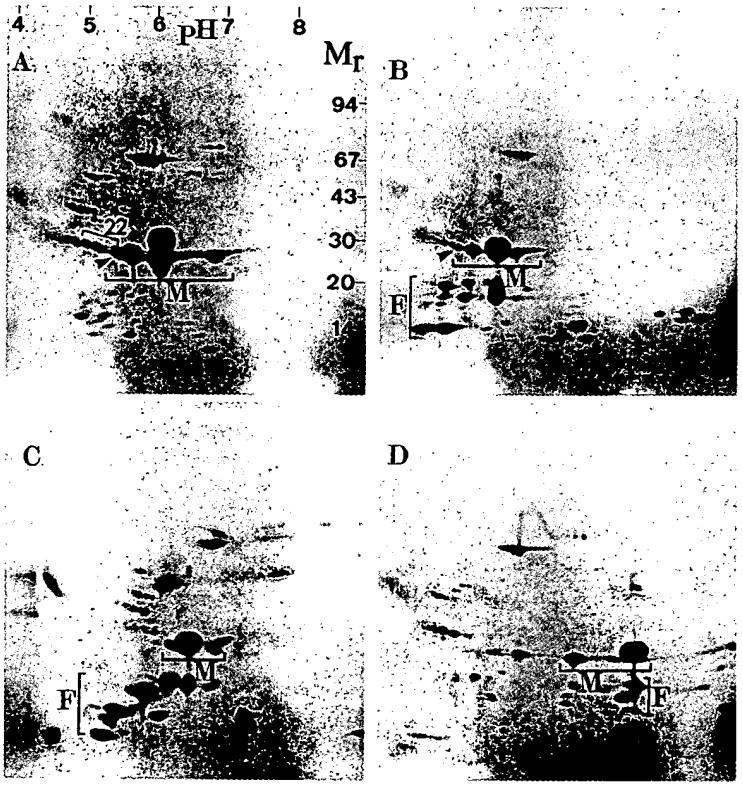

Fig. 6. 2-DE of Bence Jones proteinuria. The patterns correspond to urine (100 μg protein) from four myeloma patients analysed under reducing conditions and detected with CBB. Note: (i) the variable proportion of light chain monomer (M) and fragment (F); (ii) the charge and size heterogeneity of the light chain species; and (iii) the anodal isoforms of the monomer may co-electrophorese with the cathodal isoforms of α_1-microglobulin (arrowheads; A, B).

Tamm–Horsfall mucoprotein and γ-carboxyglutamic acid-deficient nephrocalcin) are unable to inhibit the crystallisation of calcium oxalate. Thus, differences in urinary protein composition may distinguish stone from non-stone-forming individuals [65–67]. Morse and Resnick [68] used 2-DE to investigate the precipitation of urinary proteins upon crystallisation of calcium oxalate in vitro. They demonstrated selective precipitation of α_1-microglobulin and an unidentified protein (pI 6.4; M_r 22 000) but lower than expected amounts of albumin and the immunoglobulin LCs. The urinary protein profiles pre- and post-crystallisation were virtually identical suggesting that the amount of protein precipitated was minimal [68]. In a follow up study, pooled lyophilised urinary protein of idiopathic recurrent calcium oxalate stone-formers (with or without renal calculi in situ) was compared with that from healthy controls [37]. The protein load (80 μg) was excessive for silver staining but the authors discerned seven proteins (pI 5.5–8.0; M_r 18 500–43 000) unique to the stone-formers (with or without renal calculi in situ) and two additional proteins (including α_1-acid glycoprotein) that were increased in amount relative to the controls [37].

3.2.5. Cadmium toxicity

Cadmium exposure is primarily an occupational hazard but alternative sources include cigarette smoking and the proximity of municipal incineration plants. Chronic low level exposure leads to damage of the renal proximal tubules and urinary elevation of β_2-microglobulin and retinol binding protein [69]. We have used 2-DE to compare the urinary protein profiles of workers occupationally exposed to cadmium with healthy controls [8]. The study demonstrated in the cadmium exposed a selective increase in

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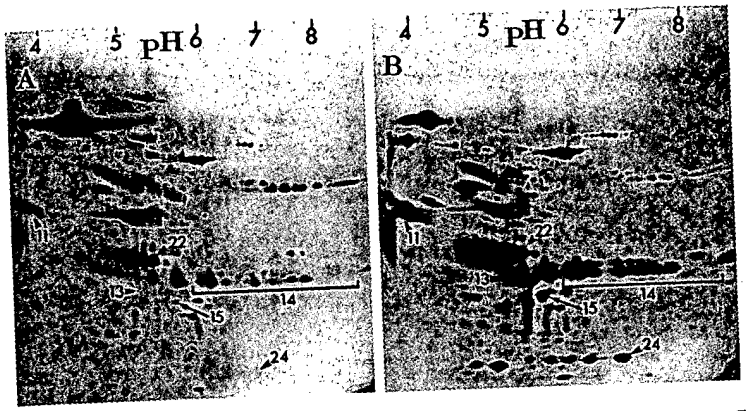

Fig. 7. 2-DE of urine (75 μl) from controls (A) and workers occupationally exposed to cadmium for 5–24 years (B). In each case, the patterns correspond to pooled samples (n=10) analysed under reducing conditions and detected with silver. Proteins elevated in the cadmium exposed include: (11) α_1-acid glycoprotein (orosomucoid); (13) apolipoprotein A-I; (14), Ig λ, κ light chains; (15) retinol binding protein; (22) α_1-microglobulin; and (24) β_2-microglobulin.

the intensity of low molecular weight polypeptides [8] including α_1-acid glycoprotein, apo A-I, immunoglobulin light chains, retinol binding protein, α_1-microglobulin and β_2-microglobulin. (Fig. 7). More recently, Myrick et al. [18,70] used 2-DE and computerised gel image analysis (Visage 2000™ System, Bio Image Products) to study urine from workers occupationally exposed to cadmium and to correlate the urinary protein patterns with the urinary cadmium (UCD) levels as determined by atomic absorption spectroscopy. Multiple hypothesis testing (regression analysis of matched 2-DE proteins with UCD levels) revealed 14 potential biomarkers for cadmium exposure but their specificity was not investigated [18].

3.2.6. *Rheumatoid arthritis (RA)*

Clark et al. [6] used 2-DE to analyse the urine of patients with RA (n=11) as compared to healthy controls (n=8) and demonstrated three high molecular weight (and one low molecular weight) spot clusters in RA but not controls. However, the patterns appear atypical relative to other reports.

3.2.7. *Duchenne muscular dystrophy (DMD)*

Frearson et al. [7] used 2-DE to compare the protein patterns of urine from healthy individuals and patients with neuromuscular (or non-muscular) diseases and detected a protein spot C (pI 5.3; M_r 26 000) associated with DMD but common to other neuromuscular diseases.

3.2.8. *Pregnancy-induced hypertension*

Clark et al. [29] used 2-DE to analyse proteinuric urines from pregnant females with or without hypertension. The latter gave patterns which were typically normal, the intensity of the spots simply increasing in proportion to the protein content [29]. In contrast, the hypertensives demonstrated a wide range of patterns (unrelated to protein content and sometimes varying from day to day) including abnormalities with a predominance of low molecular weight proteins – attributed to abnormal protein metabolism and modified tubular reabsorption in pre-eclampsia [29].

4. Recent developments

Semi-automated electrophoretic systems are now available for 2-DE which incorporate pre-cast gels to enhance reproducibility and minimise health and safety risks. The PhastSystem® (Pharmacia Biotech) has a very small gel format with a low sample volume and has not been adopted for 2-DE of human urine. The Multiphor® system (Pharmacia Biotech) is a relatively simple but expensive 2-DE method using rehydrated Immobiline® drystrips in the first dimension and precast gels with gel buffer strips for flat bed SDS–PAGE in the second dimension. We have adopted this method for 2-DE of basic LCs in BJP as the immobilised pH gradients (IPGs) prevent cathodal drift allowing detection of basic polypeptides

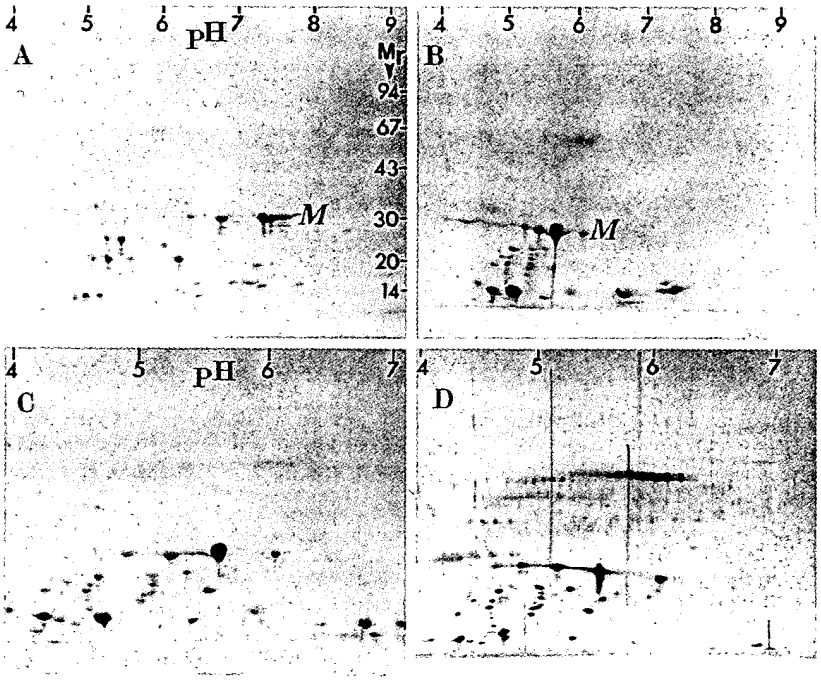

Fig. 8. Multiphor® 2-DE of Bence Jones proteinuria. The patterns correspond to myeloma patients with basic (A) or acidic free light chain (B)–(D) analysed under reducing conditions using Immobiline® strips pH 3–10 (A), (B) or pH 4–7 (C), (D) and detected with CBB (A)–(C), 30 μg protein) or silver (D), 2.5 μg protein). Note: (i) the immobilised pH gradients allow detection of basic as well as acidic light chain monomer (M); (ii) the narrower Immobiline® pH range enhances resolution of the isoforms; and (iii) the recommended silver stain (Pharmacia Biotech. PlusOne®) improves the sensitivity of detection at least 10-fold.

without recourse to NEPHGE (Fig. 8). Basic light chains may now be even better resolved using Immobiline® upto pH 12 [71]. The Multiphor®/Immobiline® system is equally suited for analysis of LCs of acidic pI, the resolution of which can be improved using Immobiline® strips of narrow pH range (Fig. 8). The accompanying silver stain is convenient to use and facilitates analysis of low protein loads (Fig. 8).

The last decade has also seen enormous advances in the development of computerised image analysis for quantitative evaluation of 2-DE patterns. Excellent commercial software packages are now available for this purpose. It is almost 20 years since Anderson et al. [72,73] first proposed the concept of a Human Protein Index whereby 2-DE would be used to separate and characterise all human proteins to establish a database for cross-reference with the Human Genome Project. The current state-of-the-art is proteome analysis whereby 2-DE, in combination with immunoblotting and microsequencing, is used to establish databases to catalogue the protein composition of the genome [74]. Such databases will contain comprehensive information on all proteins including their 2-DE co-ordinates, amino acid sequence and identity together with display and search facilities allowing cross-reference to other databases. A limited number of such web sites are currently being constructed on the Internet [52,75,76].

5. Concluding remarks

2-DE of human urinary proteins is complicated due to the lack of standardisation of sample collection, storage and processing. Further research should be directed towards the evaluation of these variables in order to improve inter-laboratory comparisons.

The choice of electrophoretic method is another variable but this is more difficult to standardise. Choice is influenced by innovation, availability and

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personal preference – an automated system may be convenient to use but prohibitively expensive with low sample throughput. Perhaps comparative 2-DE of the same urines in different key laboratories would provide a valuable insight into the relative merits of available 2-DE methods.

With respect to clinical applications commercially available 2-DE "control" urine pools would be useful as a reference material for the establishment of urinary protein databases on the Internet. There is also a general need to confirm the minimum number of clinical samples required for a statistically significant 2-DE comparison. This should now be feasible given the sophistication of computerised gel analysis.

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L Number	Hits	Search Text	DB	Time stamp
1	14	urine adj (protein or peptide).ti.	USPAT; US-PGPUB; EPO; DERWENT	2003/12/03 14:35
2	407	urine adj (protein or peptide)	USPAT; US-PGPUB; EPO; DERWENT	2003/12/03 14:55
3	11358	size adj exclusion	USPAT; US-PGPUB; EPO; DERWENT	2003/12/03 14:37
4	22	(urine adj (protein or peptide)) and (size adj exclusion)	USPAT; US-PGPUB; EPO; DERWENT	2003/12/03 14:54
5	2202	creatine adj kinase	USPAT; US-PGPUB; EPO; DERWENT	2003/12/03 14:55
6	12	(urine adj (protein or peptide)) and (creatine adj kinase)	USPAT; US-PGPUB; EPO; DERWENT	2003/12/03 15:03
7	659	superdex adj "75"	USPAT; US-PGPUB; EPO; DERWENT	2003/12/03 15:04
8	388847	serum or plasma	USPAT; US-PGPUB; EPO; DERWENT	2003/12/03 15:04
9	41	(superdex adj "75") same (serum or plasma)	USPAT; US-PGPUB; EPO; DERWENT	2003/12/03 15:04

DERWENT-ACC-NO: 1991-013490

DERWENT-WEEK: 199102

TITLE: Dysmetabolic nephropathy diagnosis in children - involves precipitating and diluting urine proteins and determining enzyme activity

INVENTOR: AKULOV, Y U S; VELTISCHCH, Y U E ; YUREVA, E A

PATENT-ASSIGNEE: MOSC PEDIATRICS RES[MOPER]

PRIORITY-DATA: 1987SU-4335636 (December 8, 1987)

PATENT-FAMILY:

PUB-NO	PUB-DATE	LANGUAGE	PAGES	MAIN-IPC
SU 1544378 A	February 23, 1990	N/A	000	N/A

APPLICATION-DATA:

PUB-NO	APPL-DESCRIPTOR	APPL-NO	APPL-DATE
SU 1544378A	N/A	1987SU-4335636	December 8, 1987

INT-CL (IPC): A61B010/00

ABSTRACTED-PUB-NO: SU 1544378A

BASIC-ABSTRACT:

In the diagnosis of dysmetabolic nephropathy in children, proteins in urine are precipitated, diluted and enzyme activity is determined. An alkali phosphatase value exceeding 5.1mm.l/min. indicates proximal position of the destructive process of the nephron, a lactate dehydrogenase value exceeding 1.1 mm l/min. indicates the distal position, and creatine kinase activity

exceeding 1.1 mm
l/min. indicates damage to the myocytes.

USE/ADVANTAGE - In medicine. The diagnosis of dysmetabolic nephropathy in
children is more accurate. Bul.7/23.2.90

CHOSEN-DRAWING: Dwg.0/0

TITLE-TERMS: NEPHROPATHY DIAGNOSE CHILD PRECIPITATION DILUTE URINE PROTEIN
DETERMINE ENZYME ACTIVE

DERWENT-CLASS: B04 P31

CPI-CODES: B04-B02C3; B04-B04A6; B04-B04B; B11-C08E; B12-K04A;

CHEMICAL-CODES:
Chemical Indexing M1 *01*
Fragmentation Code
M423 M760 M903 N102 V600 V632

Chemical Indexing M1 *02*
Fragmentation Code
M423 M750 M903 N102 V802 V811 V812 V813

Chemical Indexing M6 *03*
Fragmentation Code
M903 P723 P831 R515 R521 R612 R632

SECONDARY-ACC-NO:
CPI Secondary Accession Numbers: C1991-005984
Non-CPI Secondary Accession Numbers: N1991-010277

L6 ANSWER 5 OF 5 SCISEARCH COPYRIGHT 2003 THOMSON ISI on STN DUPLICATE 4
TI High resolution two-dimensional electrophoresis of human urinary proteins
AB The use of high resolution two-dimensional electrophoresis (2-DE) for analysis of human urine is reviewed with particular reference to methodological developments, **urinary protein** mapping and clinical applications. The methodological aspects relate to sample collection and storage, desalting and concentration of the **urinary proteins** and the choice of 2-DE and detection method. **Urinary protein** mapping includes the source of the proteins and the identification of their 2-DE positional co-ordinates. The clinical applications include 2-DE of urine from patients with renal disease, Bence Jones proteinuria, cancers of the urogenital tract, nephrolithiasis, cadmium exposure, rheumatoid arthritis, Duchenne muscular dystrophy and pregnancy-induced hypertension. Recent developments, including **proteome** analysis, are briefly discussed.
SO ANALYTICA CHIMICA ACTA, (19 OCT 1998) Vol. 372, No. 1-2, pp. 147-160.
Publisher: ELSEVIER SCIENCE BV, PO BOX 211, 1000 AE AMSTERDAM, NETHERLANDS.
ISSN: 0003-2670.
AU Marshall T (Reprint); Williams K M

L10 ANSWER 2 OF 2 CAPLUS COPYRIGHT 2003 ACS on STN DUPLICATE 2

TI Mapping of peptides and protein fragments in human urine using liquid chromatography-mass spectrometry

AB A method for the mapping of peptide mixts., heterogeneous with respect to the concn. and the size of individual peptides, was established with the aim of obtaining a comprehensive anal. of human urine peptides. Peptide extn. and fractionation were optimized to achieve a 2-step anal., using reversed-phase and ion-exchange chromatog. Highly sensitive detection of peptides was performed by coupling microbore HPLC with electrospray mass spectrometry (ESI-MS). Peptides such as urodilatin, angiotensin, and fragments of psoriasin, granulin, and uromodulin were isolated and sequenced. The procedure presented here is a tool for the anal. of complex peptide mixts. from human urine.

SO Journal of Chromatography, A (1997), 776(1), 117-124
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AU Heine, Gabriele; Raida, Manfred; Forssmann, Wolf-Georg

ADONIS Q0 244 mutation

Spiteck et al.

Anal biochem vol 258 349 (1998)